

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11) Publication number:

**0 655 505 A1**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: **94117406.2**

(51) Int. Cl.<sup>6</sup>: **C12Q 1/68, C12N 1/06**

(22) Date of filing: **04.11.94**

(30) Priority: **16.11.93 US 153325**

(43) Date of publication of application:  
**31.05.95 Bulletin 95/22**

(84) Designated Contracting States:  
**DE FR GB IT**

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(54) **Process for lysing mycobacteria.**

(57) A process for lysing Mycobacteria to liberate nucleic acids such as DNA and RNA comprises heating the Mycobacteria in an aqueous solution to lyse the Mycobacteria and release the nucleic acids, with the aqueous solution containing a chelating agent such as EDTA or EGTA in an amount effective to inhibit degradation of the released nucleic acids. Examples of Mycobacteria which can be lysed include *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. After lysis, the nucleic acid is preferably then amplified and detected. Kits useful for carrying out the present invention are also disclosed.

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### Field of the Invention

This invention relates to methods of lysing mycobacteria with heat, and methods of detecting mycobacteria employing the same.

### Background of the Invention

Mycobacteria are a large, diverse, and widely distributed family of aerobic, nonsporulating, non-motile bacilli that have a high cell-wall lipid content and a slow growth rate. Some Mycobacteria are harmless, while others such as *M. tuberculosis* are significant pathogens. Mycobacterial species are differentiated by their growth rate, pigment production, animal virulence, and biochemical reactivity.

Many detection methods for determining the presence of pathogenic Mycobacteriaceae rely on the lysis of the organisms and amplification of nucleic acids therein. However, currently available lysis procedures are expensive, laborious, time consuming and may require caustic reagents, specialized equipment, or both. This contrasts with lysis protocols for other types of cells, which generally do not require as stringent conditions for lysis.

### Summary of the Invention

A first aspect of the present invention is a process for lysing Mycobacteria such as *Mycobacterium fortuitum* and *M. tuberculosis* to liberate nucleic acid therefrom. The process comprises heating the Mycobacteria in an aqueous solution for a time and to a temperature effective to lyse the Mycobacteria, with nucleic acids being liberated or released from the Mycobacteria upon lysis. The aqueous solution contains a chelating agent (e.g., EDTA, EGTA) in an amount effective to inhibit degradation of the nucleic acids released from the Mycobacteria upon lysis thereof. The heating step may be followed by amplifying and/or detecting the nucleic acid released.

A particular embodiment of the foregoing method comprises combining a biological sample suspected of containing Mycobacteria with an aqueous solution containing a chelating agent to form a lysis solution, then heating the lysis solution for a time and to a temperature effective to lyse Mycobacteria present therein. The solution contains the chelating agent in an amount sufficient to inhibit degradation of nucleic acids released from said Mycobacteria upon the lysis thereof, but insufficient to prevent subsequent amplification of a Mycobacteria nucleic acid segment in that solution. After the heating step, the step of amplifying a Mycobacteria nucleic acid segment is carried out in the lysis solution (e.g., by adding an amplification reagent to the

lysis solution and reacting the amplification reagent with the Mycobacteria nucleic acid segment).

Also disclosed herein are kits useful for detecting Mycobacteria nucleic acid in a nucleic acid sample. The kits comprise a lysis solution containing a chelating agent in an amount effective to inhibit the degradation of Mycobacteria nucleic acid upon heat lysis of Mycobacteria therein, and typically also contain a nucleic acid amplification reagent capable of amplifying a Mycobacteria nucleic acid segment. The lysis solution and the amplification reagent are typically sealed in separate containers.

### Detailed Description of the Invention

The term "mycobacteria" as used herein has its conventional meaning in the art referring to acid-fast, non-motile, rod shaped bacteria. See generally B. Davis et al., Microbiology, 724-742 (3d Ed. 1980). All generally belong to the family Mycobacteriaceae. By way of example, the mycobacteria include, but are not limited to, *Mycobacterium africanum*, *M. avium*, *M. bovis*, *M. bovis-BCG*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. leprae*, *M. microti*, *M. paratuberculosis*, *M. scrofulaceum*, and *M. tuberculosis*.

The term "nucleic acid" as used herein refers to both DNA and RNA. The present invention is particularly preferred for use with DNA as the nucleic acid.

Mycobacteria samples employed to carry out the present invention are typically obtained in the form of a sample of a biological fluid or biological tissue suspected of containing mycobacteria. Suitable biological fluids include, but are not limited to, sputum, bronchial washings, gastric washings (containing swallowed sputum), blood, milk, and lymph fluid. Suitable tissue samples include, but are not limited to, skin and other soft tissue samples (e.g., muscle, fat). As mycobacteria infect both human and animal species, the present invention is applicable to both human and veterinary diagnostic procedures, and samples may be collected from both human and animal species. For example, *M. bovis* causes tuberculosis in cattle and is transmissible to humans, and hence the present invention may be used to diagnose infection in cattle and to test cattle milk for the presence of *M. bovis* which may be transmitted to humans. Other examples are *M. avium* and *M. intracellulare*, which infect birds (e.g., chickens and pigeons) as well as swine, and hence the present invention may be used to detect such infections. Further, humans are susceptible to infection from a variety of mycobacteria, including, but not limited to, *M. tuberculosis*, *M. kansasii*, *M. avium*, *M. intracellulare*, *M. scrofulaceum* and *M.*

*fortuitum*, and the present invention may be used to detect such infections.

Where the sample suspected of containing the mycobacteria is a sputum sample, the sample may be digested with a liquifying agent such as N-acetyl-L-cystein (NALC) and sodium hydroxide prior to or during the heating step.

The sample suspected of containing the mycobacteria is, in general, heated for a time and to a temperature effective to lyse sufficient mycobacteria in the sample for subsequent detection, such as by means of nucleic acid amplification as described below. Such a lysis effective amount of heat is sufficient to liberate intracellular components such as DNA, RNA, and the like, but insufficient to destroy or render the desired intracellular component unsuitable for subsequent use (e.g., unsuitable for use as a substrate for DNA amplification, hybridization, and/or detection). Typically, the sample is heated for a time of 5 minutes to one hour, or more typically from 10 to 20 minutes and to a temperature of from 50°C to 150°C, or more typically from about 70°C to about 120°C, depending on the nature of the sample. The only limitation is that the intracellular component to be detected, typically nucleic acid, is not destroyed by the heat. Heating may be carried out by any suitable means, including, but not limited to, water baths, microwaves, ovens, flames, and the like.

Any suitable lysis solution can be used for heating the sample suspected of containing the mycobacteria. In general, the lysis solution may be water, but can also be a suitable buffer such as tris-buffered saline (e.g., 50 mM Tris-HCl, 150 mM NaCl, pH 8.0), phosphate-buffered saline (e.g., 50 mM sodium phosphate, 150 mM NaCl, pH 8.0), polymerase chain reaction buffer (e.g., 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), REACT6™ buffer solution obtained from Bethesda Research Labs (50 mM Tris-HCl, pH 7.1, 50 mM NaCl, 50 mM KCl, 6 mM MgCl<sub>2</sub>), sodium phosphate solution (pH 5.0 to 12.0), TRIZMA™ 9.0 solution from Sigma Chemical Co. (trishydroxymethylamine). The solution may optionally contain detergent such as 0.5% Tween 20 and 0.5% Nonidet p-40. The heated sample can optionally be centrifuged, making available the supernatant and pellet for subsequent use.

The lysis solution contains a chelating agent in an amount effective to combat degradation of nucleic acid after release into the lysis solution, but preferably insufficient to interfere with subsequent detection or amplification steps. The precise amount of chelating agent will depend upon the particular chelating agent employed and lysis solution, but is typically from about 1 or 10 mM up to about 50 or 100 mM. Examples of suitable chelating agents include, but are not limited to, EDTA

(ethylene diamine tetraacetic acid) and EGTA (ethyleneglycol-bis(β-aminooethylether)-N,N,N,N-tetraacetic acid), diaminocyclohexan-tetraacetic acid (CDTA), o-phenanthroline, and salicylic acid. For EDTA, the amount included in the lysis solution is preferably about 5 mM to 15 mM, and most preferably about 10 mM. For EGTA, the amount included in the lysis solution is preferably about 1 or 2 mM to 25 or 50 mM, and most preferably from about 1 or 2 mM to 10 mM.

Once the sample is heated, subsequent use of the intracellular components can include amplification, detection, and the like. Further steps may include purification of DNA, such as by organic extraction (e.g., phenol/chloroform extraction) or solid phase adsorption onto silica surfaces such as glass or diatoms.

The process of the present invention liberates DNA and RNA from organisms in single-stranded form, enabling their direct subsequent use in an amplification or detection procedure which requires the DNA or RNA to be in single-stranded form.

Detection of mycobacteria nucleic acid (e.g., DNA or RNA) can be carried out with or without prior amplification, as discussed below. Detection may be carried out by means of Southern Blot analysis, electrophoretic gel visualization, and the like. Detection may be carried out by hybridizing a nucleic acid probe which selectively binds to mycobacteria nucleic acid thereto, which probe is labelled by conjugating a suitable detectable group thereto.

Probes can be labelled and detected by several methods. For example, probes can be radiolabelled and detected by autoradiography. Labels suitable for autoradiography include, but are not limited to, <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, and <sup>32</sup>P. Other detectable groups include ligands, fluorophores, chemiluminescent agents, electrochemical via sensors, enzymes such as horseradish peroxidase, antibodies, biotin, avidin, radionucleotides, enzyme inhibitors, co-enzymes, luciferins, paramagnetic metals, and spin labels. The choice of label dictates the manner in which the label is bound to the probe, and such binding can be carried out in accordance with known techniques.

Amplification of a selected, or target, nucleic acid sequence may be carried out by any suitable means. See generally D. Kwok and T. Kwok, *Am. Biotechnol. Lab.* 8, 14-25 (1990). Examples of suitable amplification techniques include, but are not limited to, polymerase chain reaction, ligase chain reaction, strand displacement amplification, transcription-based amplification (see D. Kwok et al., *Proc. Natl. Acad. Sci. USA* 86, 1173-1177 (1989)), self-sustained sequence replication (see J. Guatelli et al., *Proc. Natl. Acad. Sci. USA* 87, 1874-1878 (1990)), and the Qβ replicase amplification system

(see P. Lizardi et al., *BioTechnology* 6, 1197-1202 (1988)). All such techniques generally involve at least one amplification probe, or pair of amplification probes, which serves as the amplification reagent and specifically bind to the Mycobacteria nucleic acid segment to be amplified. The amplification reagent is then reacted (e.g., cyclically reacted) with the nucleic acid segment to be amplified to effect amplification thereof.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Patents Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosure of all U.S. Patent references cited herein are to be incorporated herein by reference).

Ligase chain reaction (LCR) is carried out in accordance with known techniques. See, e.g., R. Weiss, *Science* 254, 1292 (1991). In general, the reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. Each pair together completely overlaps the strand to which it corresponds. The reaction is carried out by, first, denaturing (e.g., separating) the strands of the sequence to be detected, then reacting the strands with the two pairs of oligonucleotide probes in the presence of a heat stable ligase so that each pair of oligonucleotide probes is ligated together, then separating the reaction product, and then cyclically repeating the process until the sequence has been amplified to the desired degree.

Strand displacement amplification (SDA) is also carried out in accordance with known techniques. See G. Walker et al., *Proc. Natl. Acad. Sci. USA* 89, 392-396 (1992); G. Walker et al., *Nucleic Acids Res.* 20, 1691-1696 (1992). SDA may be carried out with a single amplification primer or a pair of amplification primers, with exponential amplification being achieved with the latter. In general, SDA amplification primers comprise, in the 5' to 3' direction, a flanking sequence (the DNA sequence of which is noncritical), a restriction site for the restriction enzyme employed in the reaction, and an oligonucleotide sequence (e.g., an oligonucleotide probe of the present invention) which hybridizes to the target sequence to be amplified and/or detected. In addition, the step of first digesting the DNA sample to form a restriction fragment can be eliminated by exploiting the strand displacing activity of the DNA polymerase and adding a pair of "bumper" primers which bind to the substrate at a flanking position 5' to the position at which each amplification primer binds.

A kit for detecting mycobacteria nucleic acid in a nucleic acid sample contains one or more of (a) a lysis solution containing a chelating agent in a

concentration as described above for obtaining a released nucleic acid sample from a mycobacteria sample, and/or (b) a hybridization solution for enabling hybridization between probes and the nucleic acid sample. Probes may be provided either suspended in the solution or provided separately in lyophilized form. One example of a suitable hybridization solution is a solution comprised of 6x SSC (0.9M sodium chloride, 0.09 M sodium citrate, pH 7.0), 0.1M EDTA pH 8.0, 5x Denhardt's solution [0.1% (w/v) Ficoll Type 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin], and 100 µg/ml sheared, denatured salmon sperm DNA, commercially available from Bethesda Research Laboratories, Gaithersburg, MD 20877 USA under Catalog No. 5565UA. See also T. Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 387-388 (1982)(Cold Spring Harbor Laboratory). The components of the kit are typically packaged together in a common container (e.g., a container sealed with a frangible seal). The kit typically includes instructions for carrying out the method of the present invention as described herein (e.g., as a printed instruction sheet of printed on the package or container). Additional optional components of the kit, depending on the assay format to be employed, include one or more amplification probes or a pair of amplification probes for amplifying a Mycobacteria nucleic acid segment in the sample for subsequent detection with a detection probe, one or more detection probes for detecting a mycobacteria nucleic acid segment (with or without amplification thereof), and means for carrying out a detecting step (e.g., a probe of the invention labeled with a detectable marker and optionally an enzyme substrate when the detectable marker is an enzyme).

In use, in a preferred embodiment of the invention, the biological sample suspected of containing Mycobacteria is added to the lysis solution and heated to effect lysis and release of nucleic acid. An amplification reagent is then added directly to the lysis solution and the amplification reaction carried out in the lysis solution. The amplification reagent may be in any suitable physical form, such as carried by an aqueous solution, or in dried, lyophilized, or freeze-dried form. The ability to amplify directly in the lysis solution greatly simplifies the overall detection procedure and helps reduce potential contamination of the reaction with amplicons from previous reactions. The Mycobacteria nucleic acid segment which was amplified can then be detected by any suitable means (the possibility of contamination with amplicons being less critical after the amplification step).

The present invention is explained in greater detail in the following Examples, in which "mg" means milligrams, "ml" means milliliters, "µl"

means microliters, "mM" means milliMolar, "Kb" means kilobases, and temperatures are given in degrees centigrade. These Examples are illustrative of the present invention, and are not to be taken as limiting thereof.

#### Example 1

##### Heat Lysis of Mycobacteria DNA Produces Low Molecular Weight Fragments

The purpose of this Example is to show that lysis of mycobacteria by heat produces fragmented, low molecular weight DNA. Materials employed in this Example were: Lambda phage DNA standards (obtained from Bethesda Research Laboratories): *Mycobacterium fortuitum* (Heat-inactivated, BACTEC™-cultured), approximately 10<sup>8</sup> organisms/ml; 1% agarose gel; and ethidium bromide.

This example was carried out as follows: One ml of *M. fortuitum* was concentrated by microcentrifugation for 5 minutes and the resulting bacterial pellet was resuspended in 100 µl of 25 mM KPO<sub>4</sub>, pH 7.5 ("buffer"). This was the stock solution from which the following reactions were prepared: (1) 20 µl *M. fortuitum* + 2 µl buffer; (2) 20 µl *M. fortuitum* + 2 µl Lambda DNA standards; (3) 2 µl Lambda standards + 20 µl buffer; (4) 2 µl Lambda standards + 20 µl *M. fortuitum*; (5) 20 µl *M. fortuitum* + 2 µl Lambda standards; (6) 2 µl Lambda standards + 20 µl buffer.

The foregoing six reaction preparations were treated as follows: preparations 1-3 were heated for 15 minutes at 95 °C; preparation 4 was heated 15 minutes at 95 °C, cooled, and then 2 µl Lambda DNA added, and preparations 5 and 6 were not heated. The samples were then microcentrifuged briefly and analyzed on an ethidium stained 1% agarose gel. Reactions 1 and 2 showed DNA fluorescence in the size range of 2.3-0.1 kb. No discrete higher molecular weight bands were seen except for a faint band at about 9kb. Reactions 3-6 showed discrete DNA banding at 23.1, 9.9, 6.6, 4.4, 2.3, and 2.0 Kb, as expected for the molecular weight standards.

Intact mycobacteria genomic DNA should run at about 23 kb on an agarose gel. However, when treated as described above, there was mostly low (≤2kb) molecular weight DNA released from the mycobacteria. When lysis occurred in the presence of the lambda DNA, it was also degraded, thus indicating the process of degradation was not specific for the mycobacterial DNA. Reactions in which the lambda DNA and mycobacteria were not simultaneously heated did not show degradation of the Lambda DNA, suggesting that heat and the presence of mycobacteria or its media activated a DNA degradation process which was not specific

for mycobacterial DNA.

#### Example 2

##### Effect of Chelation, Reduction and Time on Heat Lysis of Mycobacteria DNA

The purpose of this example was to determine whether chelation, reduction or time affects the degradation of mycobacterial DNA during lysis. Materials employed were as for example 1 above, also including the reducing agent dithiothreitol (DTT) and ethylenediamine tetraacetic acid (EDTA). In brief, three 1 ml aliquots of *M. fortuitum* culture were microcentrifuged for 5 minutes. Each bacterial pellet was resuspended in either: (A) 100 µl of 25 mM KPO<sub>4</sub> solution, (B) 100 µl of 25 mM KPO<sub>4</sub> solution containing 50 mM EDTA, or (C) 100 µl of 25 mM KPO<sub>4</sub> solution containing 50 mM DTT. Twenty µl of each stock *M. fortuitum* solution was then given one of the following four treatments:

(I) heated at 95 °C for 15 minutes;

(II) 2 µl Lambda DNA were added, then the reaction was heated at 95 °C for 15 minutes;

(III) heated at 95 °C for 15 minutes, then 2 µl Lambda DNA were added; or

(IV) 2 µl Lambda DNA were added but the reaction was not heated. Control reactions containing only Lambda DNA were set up as:

(V) 2 µl Lambda DNA in 20 µl A or 20 µl B or 20 µl C and heated at 95 °C for 15 minutes.

After treatment, the reaction solutions were stored overnight at 4 °C.

On the following day after the reactions described above, the reaction solutions were analyzed on ethidium-stained 1% agarose gels. In treatment I (heating alone), the presence of EDTA (B) resulted in DNA with a discrete molecular weight at about 20-23 kb. The other two conditions (DTT or buffer alone) produced DNA as a low molecular weight smear of size less than the 2.3 kb size marker. DTT was expected to bind free radicals, or in the alternative the sulfur therein was expected to bind metal ions, hence the lack of activity of DTT was a surprise.

Treatment II (Lambda DNA and heating) showed that the presence of EDTA conserved the Lambda DNA standards at the appropriate molecular weight while in buffer alone or in the presence of DTT the Lambda DNA markers appeared as a low molecular weight smear. In all of the other treatments, the Lambda DNA standards migrated as the appropriate sizes.

These data show that addition of 50 mM EDTA reduces the appearance of low molecular DNA from *M. fortuitum* and the apparent cleavage of Lambda DNA.

### Example 3

#### Titration of the Effect of Chelators on DNA Degradation During *M. fortuitum* Lysis

Materials employed in this example are the same as described in Examples 1 and 2 above, including EDTA (ethylene diamine tetraacetic acid) and EGTA (ethyleneglycol-bis(B-aminoethylether)-N,N,N,N tetraacetic acid).

Three 1 ml aliquots of BACTEC™-cultured *M. fortuitum* were microcentrifuged for 5 minutes. The resulting pellets were resuspended individually with 100 microliters of 25 mM KPO<sub>4</sub> solution, pH 7.5 and then pooled. Reactions were set up as 20  $\mu$ l *M. fortuitum* + 2  $\mu$ l chelator (either EGTA or EDTA) at one of the following tenfold serial dilutions - 0.5; 0.05; 0.005; 0.0005; 0.00005 or 0.000005 M. (The control consisted of adding 2  $\mu$ l of 25 mM KPO<sub>4</sub>). The reactions were then heated to 95°C for 15 minutes and the debris was pelleted by microcentrifugation for one minute. Each reaction received 4  $\mu$ l of type II sample buffer (described in J. Sambrook et al., Molecular Cloning: A Laboratory Manual, table 6.3 (2d Ed. 1989)(6x buffer is: 0.25% bromophenol blue; 0.25% xylene cyanol FF; 15% Ficoll (Type 400; Pharmacia) in water)) and 15  $\mu$ l was analyzed by ethidium-stained 1% agarose gels. The resulting gel profiles indicated that both chelators produce inhibition of the DNA breakdown, but EGTA seemed better able to protect the genomic DNA. This suggests that the mechanism of DNA cleavage during *M. fortuitum* lysis is not magnesium-dependent, since EGTA is not a good chelator for Mg<sup>2+</sup>.

### Example 4

#### Heat Lysis of *M. fortuitum* DNA in Sputum in the Presence or Absence of Chelating Agents

The purpose of this Example was to determine whether lysis of *M. fortuitum* in sputum produces cleaved DNA, and whether that cleavage is stopped by chelating agents.

Materials employed in this Example are essentially the same as those described for Examples 1-3 above, and also included processed clinical sputum samples from the Veterans Administration Hospital, Durham, North Carolina, USA. (Accession numbers 903, 909, 904, 910, 786, 783, 987, 899, 790, 906, 901, 984, 898, 791, and 902).

One milliliter aliquots of BACTEC™-cultured *M. fortuitum* (about 10<sup>8</sup> organisms/ml) were transferred into 1.5 ml SARSTEDT™ screw cap tubes and pelleted by microfugation for 5 minutes. The supernatants were discarded and about 1 ml of pooled, processed sputum was added along with

about 20 mg of cotton. The samples were then mixed by hand and vortexed, then microcentrifuged for 5 minutes. The supernatants were again discarded and the pellets resuspended in 1 ml of 25 mM KPO<sub>4</sub> at pH 7.5 containing 0, 0.5, 5 or 50 mM concentrations of chelator (EDTA or EGTA). After mixing, the tubes were centrifuged again and the supernatants discarded. The tubes were then heated for 15 minutes at 95°C. About 100  $\mu$ l of supernatant was aspirated from each tube and given an approximately 2 second microcentrifugation. 20  $\mu$ l of those supernatants were then analyzed by electrophoresis in an ethidium-stained 1% agarose gel.

Results from these experiments were consistent with the findings reported above. During lysis of the mycobacteria, the DNA was cleaved and in the presence of either chelator the genomic-sized DNA was retained. The best results (i.e., with the most intact genomic DNA remaining) were observed with 0.5 and 5 mM EGTA.

### Example 5

#### Compatability of Heat Lysis in the Presence of Chelator with a DNA Amplification Reaction

The purpose of this example was to demonstrate that addition of EGTA to an *M. tuberculosis* lysis reaction is compatible with the polymerase chain reaction (PCR).

Materials employed with this example were essentially as described above, except that *M. tuberculosis* was used rather than *M. fortuitum*, and PCR reagents were also used in accordance with standard techniques. In brief, 1000 *M. tuberculosis* cells were spiked into 1 ml of 25 mM KPO<sub>4</sub> at pH 7.5 and heated for 15 minutes at 95°C. 50  $\mu$ l reactions were set up, to which were added 1  $\mu$ l of 0.5, 0.05, 0.005, 0.0005 Molar EGTA solution or H<sub>2</sub>O. Then, 50  $\mu$ l of PCR buffer containing primers for the IS6110 target segment (see D. Thierry et al., IS6110, an IS-like element of *Mycobacterium tuberculosis* complex, *Nucleic Acids Res.* 18, 188 (1990)) was added and PCR was carried out in accordance with standard procedures.

It was found that 10 mM EGTA inhibited the PCR reaction slightly, but other concentrations were not inhibitory. A 1 mM concentration, which did not inhibit amplification, is consistent with the concentration observed to preserve intact DNA in Example 4 above. Therefore, lysis of *M. tuberculosis* in the presence of EGTA releases intact, genomic-sized DNA, and at concentrations of EGTA below about 10 mM will not appreciably inhibit the PCR amplification.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the

following claims, with equivalents of the claims to be included therein.

#### Claims

1. A process for lysing Mycobacteria to liberate nucleic acid therefrom, comprising:  
     heating the Mycobacteria in an aqueous solution for a time and to a temperature effective to lyse the Mycobacteria,  
     said aqueous solution containing a chelating agent in an amount effective to inhibit degradation of nucleic acids released from said Mycobacteria upon lysis thereof.
2. A process according to Claim 1, wherein said chelating agent is selected from the group consisting of ethylene diamine tetraacetic acid (EDTA) and ethyleneglycol-bis(B-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA).
3. A process according to Claim 1, wherein said heating step is carried out for a time of five minutes to one hour and at a temperature of from 50° to 150° C.
4. A process for detecting Mycobacteria in a sample suspected of containing the same, comprising:  
     combining said sample with an aqueous solution containing a chelating agent to form a lysis solution;  
     heating said lysis solution for a time and to a temperature effective to lyse Mycobacteria present therein, said solution containing said chelating agent in an amount sufficient to inhibit degradation of nucleic acids released from said Mycobacteria upon the lysis thereof; and then, in said lysis solution,  
     amplifying a Mycobacteria nucleic acid segment with a DNA amplification reaction.
5. A process according to Claim 4, wherein said amplifying step is carried out by adding an amplification reagent to said lysis solution, and reacting said amplification reagent with said Mycobacteria nucleic acid segment.
6. A process according to Claim 4, wherein said amplification reagent is selected from the group consisting of polymerase chain reaction, ligase chain reaction, strand displacement amplification, transcription-based amplification, self-sustained sequence replication, and Q $\beta$  replicase amplification reagents.
7. A process according to Claim 4, wherein said amplifying step is followed by the step of detecting said Mycobacteria nucleic acid segment.
8. A kit useful for detecting Mycobacteria nucleic acid in a nucleic acid sample, comprising:  
     (a) a lysis solution containing a chelating agent in an amount effective to inhibit the degradation of Mycobacteria nucleic acid upon heat lysis of Mycobacteria in said lysis solution; and  
     (b) a nucleic acid amplification reagent capable of amplifying a Mycobacteria nucleic acid segment.
9. A kit according to Claim 8, wherein said chelating agent is selected from the group consisting of ethylene diamine tetraacetic acid (EDTA) and ethyleneglycol-bis(B-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA).
10. A kit according to Claim 8, wherein said amplification reagent is selected from the group consisting of polymerase chain reaction, ligase chain reaction, strand displacement amplification, transcription-based amplification, self-sustained sequence replication, and Q $\beta$  replicase amplification reagents.



European Patent  
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## EUROPEAN SEARCH REPORT

Application Number  
EP 94 11 7406

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	MOLECULAR AND CELLULAR PROBES, vol.5, no.5, October 1991, ACADEMIC PRESS, LONDON, GB; pages 385 - 395 V. SRITHARAN AND R.H. BARKER JR. 'A simple method for diagnosing M. tuberculosis infection in clinical samples using PCR' * page 388, left column, line 2 - line 16 * * page 389, left column, line 17 - line 20 *	1-10	C12Q1/68 C12N1/06
X	--- J. CLIN. MICROBIOL., vol.31, no.7, July 1993, AM. SOC. MICROBIOL., WASHINGTON, DC,US; pages 1811 - 1814 J. V. IRALU ET AL. 'Diagnosis of Mycobacterium avium bacteremia by polymerase chain reaction' * page 1811, right column, line 11 - page 1812, left column, line 15 *	1-10	
X	EP-A-0 556 521 (BECTON DICKINSON AND COMPANY) 25 August 1993 * the whole document *	1-3,8-10	C12Q C12N
X	EP-A-0 552 571 (BECTON DICKINSON AND COMPANY) 28 July 1993 * page 13, line 1 - page 14, line 3; tables I,II * --- -/--	1-3,8-10	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 28 February 1995	Examiner Hornig, H
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

EPO FORM 150 (03.92) (P4/C01)





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X	JOURNAL OF BACTERIOLOGY, vol.104, no.2, November 1990, AM. SOC. MICROBIOL., BALTIMORE, US; pages 1020 - 1021 Y MIZUGUCHI AND T. TOKUNAGA 'Method for isolating deoxiribonucleic acid from mycobacteria' * page 1020, left column, line 29 - line 38 *	1,2	
A	EP-A-0 547 789 (BECTON DICKINSON AND COMPANY) 23 June 1993 * the whole document *	1-10	
A	EP-A-0 520 756 (BECTON DICKINSON AND COMPANY) 30 December 1992 * the whole document *	1-10	
A	MOLECULAR AND CELLULAR PROBES, vol.5, no.3, June 1991, ACADEMIC PRESS, LONDON, GB; pages 215 - 219 B.B. PLIKAYTIS ET AL. 'Differentiation of Mycobacterium tuberculosis and Mycobacterium bovis by a polymerase chain reaction' * page 216, left column, line 10 - right column, line 13 *	1-10	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	J. CLIN. MICROBIOL., vol.28, no.2, February 1990, AM. SOC. MICROBIOL., WASHINGTON, DC,US; pages 319 - 323 K. DIX ET AL. 'Species-specific oligonucleotide probes for the identification of periodontal bacteria' * page 319, right column, line 10 - line 31 *	1,2,8,9	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 28 February 1995	Examiner Hornig, H
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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